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(54) Title: METHODS OF USING INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS

(57) Abstract

The invention relates to methods of using insulin-like growth factor binding proteins ("IGFBPs"), including IGFBP-1 or modified forms of IGFBP-1 as therapeutic agents. Modified forms include IGFBP-1 attached to a polymer or two IGFBP-1 molecules attached to opposite ends of a polymer. The methods involve administering IGFBPs, including IGFBP-1 or a modified form of IGFBP-1 to a patient having an IGF associated condition sufficient to cause a therapeutic effect. The invention also relates to non-phosphorylated IGFBPs useful in the methods.

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METHODS OF USING INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS

FIELD OF THE INVENTION

This invention relates to uses of insulin-like growth factor binding protein 1 (IGFBP-1 or BP-1) as a therapeutic agent.

BACKGROUND OF THE INVENTION

Circulating insulin-like growth factors I and II (IGF-I and IGF-II) are 7 kDa proteins that are related in structure to each other and to insulin. IGF-I and IGF-II are growth and differentiation factors for most cells in the body and are present at high concentrations in serum (about 300 ng/ml for IGF-I and 1000 ng/ml for IGF-II). Circulating levels of IGF-I are determined primarily by growth hormone (GH), which stimulates the liver to make IGF-I. Most of the growth-promoting effects of growth hormone are believed to be mediated by IGF-I.

Tissue IGF's also exist. Tissue IGF-I has a larger apparent molecular mass (approximately 26 kDa), as determined by gel chromatography, than circulating IGF. Rom, W.N. et al., J. Clin. Invest., 82: 1685-1693 (1988).

IGF-I and IGF-II have been shown to play a role in a large number of disease conditions. These include, for example, breast cancer, colon cancer, lung cancer, ovarian cancer, osteosarcoma, glioma, liver cancer, prostate cancer, rhabdomyosarcomas, restenosis, acromegaly, obesity, tumor-induced hypoglycemia, pulmonary fibrosis, diabetic nephropathy and diabetic retinopathy.

The roles of insulin-like growth factors in human tumors are discussed in Daughaday, Endocrinol. 127:1-4 (1990). For example, IGF-I and IGF-II are believed

to function as autocrine or paracrine growth factors for a variety of human cancers, including breast cancers, colon cancers, lung cancers, ovarian cancers, osteosarcomas, neuroblastomas, gliomas, Wilm's tumors, and rhabdomyosarcomas as reported in Cullen et al., Cancer Investigation, 9:443-454 (1991). A wide variety of primary tumors have been shown to overexpress IGF-I or IGF-II and to express receptors for these growth factors, as reported in Yee et al, Cancer Res., 48:6691-6696 (1988) and Osborne et al, Mol. Endocrinol. 3:1701-1709 (1989). Numerous in vitro studies have shown that human cell lines derived from some of the above cancers proliferate in response to IGF-I and IGF-II. In some cases the cell lines have been shown to produce IGF-I or IGF-II and to possess cell surface receptors for IGF-I and IGF-II. In some instances, particularly in breast cancer, stromal cells surrounding the tumor have been shown to secrete IGF-I and IGF-II, resulting in a paracrine growth relationship.

Breast, lung and colon cancers are the three most common cancers in the U.S., affecting nearly 500,000 people. The strongest evidence for a role of IGF-I and IGF-II in growth of these cancers comes from experiments showing that antibodies to the IGF-I receptor block tumor formation in nude mice by a number of cell lines derived from human tumors of these types.

Analyses of biopsies from human colon cancers showed overexpression of IGF-II 10- to 50-fold in 40% of the tumors analyzed, as reported in Tricoli et al., Cancer Research, 46:6169-6173 (1986). The level of IGF-II overexpression correlated with the degree of invasion of the bowel wall.

IGF-I can mediate autocrine proliferation of human small cell lung cancer cell lines NCI-H345 and NCI-N417, as reported in Nakanishi, et al., J. Clin. Invest., 82:354-359 (1988).

Ovarian cancer cell lines OVCAR-3, OVCAR-7 and PEO4 express IGF-I mRNA. Primary and metastatic ovarian cancer tissues also express IGF-I mRNA as well as Type I IGF receptor mRNA, as reported in Yee, et al, Cancer Res., 51:5107-5112 (1991).

Both normal and neoplastic bone cells secrete and respond to IGF-I, as reported in Blatt, et al Biochem. Biophys. Res. Commun. 123:373-376 (1984) and Canalis. J. Clin. Invest. 66:709-719 (1980).

IGF-II mRNA expression is developmentally regulated in liver tissue. Increased IGF-II mRNA levels have been detected in liver cancers of woodchucks, humans and rats, as reported in Cariani, et al., J. Hepatology, 13:220-226 (1990).

Three human prostatic cancer cell lines, PC-3, DU-145, and LNCa.FGC, produce substantial amounts of IGF-I and display constitutively auto-phosphorylated IGF-I receptors. Pietrzkowski, Z. et al., Cancer Research, 53: 1102-1106 (1993). Pietrzkowsky et al. report that growth of all three of these cell lines was inhibited by an antisense oligodeoxynucleotide to IGF-I receptor RNA or by peptide analogs of IFG-I that compete with IGF-I binding to its receptor.

Rhabdomyosarcoma is the most common soft tissue sarcoma of childhood and appears to arise from developing striated muscle-forming cells. Elevated levels of IGF-II mRNA and have been reported in rhabdomyosarcoma tumors as described in El-Badry, et al., Cell Growth & Differentiation, 1:325-331 (1990).

As indicated above, the insulin-like growth factors have been associated with certain non-cancerous disorders such as acromegaly and restenosis, for example. Acromegaly results from an excess production of growth hormone (GH). Growth hormone acts by stimulating production of IGF-I. Thus, acromegaly has been associated with increased levels of IGF-I.

Restenosis, reocclusion of the artery, occurs within 6 months in approximately 25-55% of patients who undergo angioplasty surgery. Thickening of the intimal layer of the artery is the primary cause of restenosis. Intimal thickening occurs as a result of smooth muscle cell proliferation and secretion of extracellular matrix components. It has been shown that IGF-I gene expression is induced 9-fold in the denuded artery following angioplasty surgery, as reported in Cercek et al Circulation Research, 66:1755-1760 (1990). The timing and level of IGF-I gene expression closely parallels that of smooth muscle cell proliferation. Hybridization studies indicate that the dividing smooth muscle cells are the cells that exhibit increased IGF-I gene expression, as reported in Khorsandi et al, J. Clin. Invest., 90:1926-1931 (1992). Other studies have shown that animals with low circulating IGF-I levels (due to removal of their pituitaries) have greatly reduced intimal thickening following angioplasty surgery, as reported in Khorsandi et al, Atherosclerosis, 93:115-122 (1992).

Hypogylcemia associated with certain tumors has long been known. Unusually high levels of IGF-II mRNA and of IGF-II immunoreactive peptide were observed in a leiomyosarcoma removed from a patient with recurrent hypoglycemia. Daughaday, W.H., et al., New England Journal of Medicine, Vol. 319, No. 22: 1434-1440 (1988). After the leiomyosarcoma was removed, the hypoglycemia subsided. Id.

Others studying tumor-induced hypoglycemia report finding elevated plasma IGF-II levels before the tumors were treated and prompt reduction of IGF-II levels and resolution of the hypoglycemia after the tumors were treated. Axelrod, L. and Ron, D., New England Journal of Medicine, Vol. 319, No. 22: 1477-1479 (1988).

In vivo administration of IGF-1 can also induce hypogylcemia. Lewitt, M. S., et al., Endocrinology, Vol. 129, No. 4: 2254-2256 (1991). Lewitt at al. also report that in vitro studies have shown IGFBP-1 inhibition of glucose incorporation into fatty acids of rat adipose tissue.

In pulmonary fibrosis, there is an increased number of activated alveolar macrophages and an exaggerated accumulation of fibroblasts in the alveolar walls. The fibroblasts secrete an extracellular collagenous matrix. The fibroblasts and the matrix secretions cause the alveolar walls to thicken and cause a loss of alveolar-capillary units. Activated alveolar macrophages have been shown to release an IGF-1 that signals replication of fibroblasts. Rom, W.N., et al., J. Clinic. Invest., 82: 1685-1693 (1988). Alveolar macrophages from patients with interstitial lung disorders have been shown to spontaneously secrete this IGF-I. Id.; Bitterman, P.B. et al., J. Clin. Invest., 72: 1801-1813 (1983). Current treatments for the cancerous and non-cancerous disorders include surgery, radiation, chemotherapy and hormone therapy. For example, various cancers, such as breast, lung, ovarian, colon and osteosarcomas are treated with surgery, radiation and chemotherapeutic agents. Chemotherapeutic agents used for treating these cancers include fluoropyrimidines and alkylating agents. Both of these groups exhibit significant toxicities, including, for example, myelosuppression, immunosuppression, neutropenia, gastrointestinal toxicity, renal toxicity and peripheral neuropathies. Additionally, there is no known

effective chemotherapeutic agent for treatment of liver cancer. Surgery is highly invasive and unpredictable, while radiation is non-specific at the point of localization. Hormone therapy has undesirable side effects such as unwanted hair growth and mood changes.

Antibodies to the type I IGF-I receptor have been shown to block growth of IGF-I-responsive carcinoma cell lines in vitro. Studies have shown that antibodies to the IGF-I receptor block growth of certain breast and lung carcinomas transplanted into immunodeficient nude mice as reported in Arteaga et al., J. Clin. Invest., 84:1418-1423, (1989); and Zia et al., Proc. Amer. Assoc. for Cancer Research, 33:270, Abstract 1616 (1992).

Antisense sequences to the IGF-I gene have been shown to block growth of a malignant rat glioma cell line transplanted into rats as described in Trojan et al., Proceedings of the National Academy of Science, 89:4874-4878 (1992). However, using antisense sequences for gene therapy is still in the early development stage.

Thus, a need exists for an agent which would inhibit the action of IGFs in the above cancers and disease conditions. The present invention provides such an IGF inhibitory agent, namely IGFBP-1 for the inhibition of the inappropriate action of IGFs in these disease conditions.

SUMMARY OF THE INVENTION

This invention relates to methods of using insulin-like growth factor binding proteins (IGFBPs) as a therapeutic agent to treat or prevent IGF-associated conditions. In particular embodiments of the invention, the binding protein is IGFBP-1, also termed "BP-1." This invention also relates to methods of using a modified forms of IGFBPs as a therapeutic agent. For example, modified forms of IGFBP-1 include

IGFBP-1 attached to a polymer or 2 or more IGFBP-1 molecules attached to a polymer. The methods involve administering IGFBPs, including for example, IGFBP-1 or a modified form of IGFBP-1 to a patient having an IGF associated condition sufficient to cause a therapeutic effect. For IGFBP-1, it is contemplated that a therapeutic effect can be achieved when circulating levels of IGFBP-1 range from about 0.1 μg to about 300 μg per ml in the bloodstream of the patient. Examples of conditions wherein administration of IGFBPs, in particular IGFBP-1 may be useful include treatment or prevention of breast cancer, colon cancer, lung cancer, ovarian cancer, osteosarcoma, glioma, liver cancer, prostate cancer, rhabdomyosarcomas, restenosis, acromegaly, obesity, tumor-induced hypoglycemia, pulmonary fibrosis, diabetic nephropathy and diabetic retinopathy. This invention also relates to pharmaceutical compositions containing IGFBPs, and in particular, IGFBP-1 or a modified form of IGFBP-1 and methods of treating or preventing IGF associated conditions using the pharmaceutical compositions.

DETAILED DESCRIPTION OF THE INVENTION

Inappropriate expression or utilization of IGF-I or IGF-II is a contributing factor in many disease conditions. It is contemplated that administration of IGFBPs, and in particular IGFBP-1, may be a useful therapeutic in disease conditions which are associated with inappropriate expression or utilization of IGFs, particularly IGF-I or IGF-II. Thus, the present invention is directed to methods of treating a patient having an IGF associated condition or of preventing an IGF-associated condition by administering an amount of IGFBP, including IGFBP-1 or a modified form of IGFBP-1, sufficient to cause a therapeutic effect.

Terms used throughout this specification are defined as follows:

The term "acceptable pharmaceutical carrier" refers to a physiologically-compatible, aqueous or non-aqueous solvent.

The term "IGF-I" refers to a protein containing the same amino acid sequence as naturally occurring IGF-I, or a protein containing the same amino acid sequence as naturally occurring IGF-I with the addition of an N-terminal methionine (met-IGF-I), unless otherwise specified.

The term "IGF" refers to any polypeptide that binds to the IGF type I Receptor, including, for example, IGF-I, IGF-II, (des1-3)IGF-I, met-IGF-I, insulin, and any active fragments which bind to the type I Receptor. This hormone family is described in Blundell and Humbel, Nature, 287:781-787 (1980).

The term "IGF associated condition" refers to an existing or potential adverse physiological condition which results from or is associated with an overproduction or underproduction of IGF, IGF binding protein or IGF receptor, inappropriate or inadequate binding of IGF to binding proteins or receptors and any disease in which IGFBP, particularly IGFBP-1, administration alleviates or reduces disease symptoms. An IGF associated condition also refers to a condition in which administration of IGFBPs including IGFBP-1 to a normal patient has a desired effect. Examples of IGF associated conditions include breast cancer, colon cancer, osteosarcoma, glioma, lung cancers, rhabdomyosarcomas, ovarian cancer, liver cancer, acromegaly, obesity, tumor-induced hypoglycemia, pulmonary fibrosis, restenosis, diabetic nephropathy and diabetic retinopathy.

The term "patient" refers to any animal, including humans, in need of treatment for an IGF associated condition.

The term "IGFBP" refers to any of the six known IGF binding proteins or to fragments of these binding proteins which bind to IGF. IGF-I and IGF-II circulate in blood bound to specific binding proteins of which six are now known. The binding proteins bind 95% or more of the IGFs in blood. One theory is that when bound by binding proteins, IGF-I and IGF-II are prevented from interacting with certain cell surface receptors mediating their biological functions.

IGF binding protein-1 is a 23 kDa IGF binding protein. IGFBP-1 is expressed in vivo during periods of growth arrest (e.g., starvation and diabetes), suggesting that IGFBP-1 acts as an IGF-I inhibitor. Oh et al., Endocrinol. 132:1337-1344, 1993, report IGF-I and IGF-II are essentially equipotent in their affinities for IGFBP-1.

Amniotic fluid is a natural source of IGFBP-1 and contains both phosphorylated and non-phosphorylated forms of this binding protein. Jones, J.I. et al., Proc. Natl. Acad. Sci., 88: 7481-7485 (1991). Phosphorylated BP-1 has a higher affinity for IGF-1 than the non-phosphorylated form. Jones, J.I. et al., J. Biol. Chem., 268, 2: 1125-1131 (1993). Jones et al. propose that the phosphorylated form of BP-1 is inhibitory to cell growth, while non-phosphorylated BP-1 is stimulatory to cell growth. Recombinantly produced BP-1 expressed in bacteria is non-phosphorylated and has been shown to potentiate the effects of IGF-1. Ladin, D. et al., J. Cellular Biochemistry, Supplement 17E: 127 (1993).

Data provided herein, however, demonstrates that non-phosphorylated IGFBP-1 can also be inhibitory to cell growth in vitro and in vivo. Specifically, it has been found that bacterially-derived recombinant BP-I inhibits the damaging cell growth which attends certain IGF-associated conditions.

Accordingly, the IGFBP-1 useful in the methods of the present invention can be phosphorylated or non-phosphorylated. Thus, the BP-1 useful in the present invention can be purified from natural sources such as amniotic fluid, or can be produced in accordance with recombinant procedures well known in the art. The amino amino acid sequence of mature IGFBP-1 is:

Ala-Pro-Trp-Gln-Cys-Ala-Pro-Cys-Ser-Ala-Glu-Lys-Leu-Ala-Leu-Cys-Pro-Pro-Val-Ser-Ala-Ser-Cys-Ser-Glu-Val-Thr-Arg-Ser-Ala-Gly-Cys-Gly-Cys-Cys-Pro-Met-Cys-Ala-Leu-Pro-Leu-Gly-Ala-Ala-Cys-Gly-Val-Ala-Thr-Ala-Arg-Cys-Ala-Arg-Gly-Leu-Ser-Cys-Arg-Ala-Leu-Pro-Gly-Glu-Gln-Gln-Pro-Leu-His-Ala-Leu-Thr-Arg-Gly-Gln-Gly-Ala-Cys-Val-Gln-Glu-Ser-Asp-Ala-Ser-Ala-Pro-His-Ala-Ala-Glu-Ala-Gly-Ser-Pro-Glu-Ser-Pro-Glu-Ser-Thr-Glu-Ile-Thr-Glu-Glu-Glu-Leu-Leu-Asp-Asn-Phe-His-Leu-Met-Ala-Pro-Ser-Glu-Glu-Asp-His-Ser-Ile-Leu-Trp-Asp-Ala-Ile-Ser-Thr-Tyr-Asp-Gly-Ser-Lys-Ala-Leu-His-Val-Thr-Asn-Ile-Lys-Lys-Trp-Lys-Glu-Pro-Cys-Arg-Ile-Glu-Leu-Tyr-Arg-Val-Val-Glu-Ser-Leu-Ala-Lys-Ala-Gln-Glu-Thr-Ser-Gly-Glu-Glu-Ile-Ser-Lys-Phe-Tyr-Leu-Pro-Asn-Cys-Asn-Lys-Asn-Gly-Phe-Tyr-His-Ser-Arg-Gln-Cys-Glu-Thr-Ser-Met-Asp-Gly-Glu-Ala-Gly-Leu-cys-Trp-Cys-Val-Tyr-Phe-Trp-Asn-Gly-Lys-Arg-Ile-Pro-Gly-Ser-Pro-Glu-Ile-Arg-Gly-Asp-Pro-Asn-Cys-Gln-Ile-Tyr-Phe-Asn-Val-Gln-Asn (SEQ ID NO.:1).

The amino acid sequence of the signal sequence is: Ser-Glu-Val-Pro-Val-Ala-Arg-Val-Trp-Leu-Val-Leu-Leu-Leu-Leu-Thr-Val-Gln-Val-Gly-Val-Thr-Ala-Gly (SEQ ID No.:2).

Using SEQ ID NO.:1, one skilled in the art can chemically synthesize a DNA encoding IGFBP-1. Alternatively, one skilled in the art can design oligonucleotide probes based upon SEQ ID NO.:1 to isolate a genomic DNA or mRNA and generate

a cDNA. The DNA encoding the IGBBP-1 can be used to transform a host for recombinant production.

For example, BP-1 can be expressed in E. coli BL21/DE3 using the T7 promoter system as an insoluble protein in inclusion bodies. BL21/DE3 is described by Studier, F.W., and Moffatt, B., J. Mol. Biol, 189: 113-30 (1986). Alternatively, a TAC promoter system can be employed. In E. Coli, the recombinantly expressed BP-1 is contained in the isoluble fraction. The insoluble protein is improperly folded and inactive. BP-1 can be denatured and folded into its proper conformation by dissolving the protein in 6M guanidine and a reducing agent, diluting the mixture 10-fold and allowing the BP-1 to refold overnight. IGFBP-1 contains 18 cysteine residues, all of which are believed to participate in forming disulfide bridges. Despite the large number of cysteine residues in BP-1, the protein refolds to a single major species. The refolded protein can be purified using sequential Q-sepharose and butyl-sepharose columns. The yield of purified BP-1 per 10L fermenter run is about 1.5 g.

IGFBP-1 can also be expressed in mammalian expression systems as set forth in Jones, J.I. et al., Proc. Natl. Acad. Sci., 88: 7481-7485 (1991) and in Jones, J.I. et al, J. Biol. Chem., 268, 2: 1125-1131 (1993), both specifically incorporated herein by reference. For expression in mammalian systems, a DNA encoding both the mature protein and the signal sequence should be employed. One skilled in the art can choose any appropriate vector and expression system as desired.

The therapeutic utility of IGFBPs, including IGFBP-1, may be enhanced by increasing their circulating half-lives. Increasing the molecular weight of a protein, for example by covalently bonding an inert polymer chain such as polyethylene glycol

(PEG) to the protein, is known to increase the circulating half-life of the protein in the body. See, for example, Davis et al, Biomedical Polymers: Polymeric Materials and Pharmaceuticals for Biomedical Use, p. 441-451 (1980). Covalent attachment of PEG to protein is termed herein "PEGylation." The term "PEGylated" means conjugated to polymer.

One useful method of PEGylation involves creation of a mutein having a cysteine residue available for attachment to a polymer activated with a thiol-specific reactive group. Muteins can be prepared using mutagenesis techniques well known in the art. For example, the IGFBP-1 mutein is created by replacing one or more specific amino acids with cysteine residues, or by inserting a cysteine residue between amino acids or at the N or C terminus. It is expected that such non-native cysteine residues will be "free," i.e. not involved in intramolecular disulfide bonds. Non-native cysteine residues can be substituted or inserted in regions of the IGFBP-1 molecule that are exposed on the protein's surface, and which are not involved in receptor binding or binding to IGF. One site for insertion or substitution of the cysteine may occur in the middle of the BP-1 protein. It is believed that cysteine can be substituted or inserted from amino acid 60 to 180, with the residue numbering based upon SEQ ID NO.:1. Particularly useful muteins include the substitution of a cysteine residue at positions 98 and 101 for the naturally occurring serine found at those locations.

Attachment of the inert polymer chain molecule to one or more IGFBP-1 molecules creates a further modified form of IGFBP-1, an IGFBP-1-polymer conjugate also called "PEGylated IGFBP-1". Coupling of thiol-specific reactive groups to polymers is discussed PCT Application Publication No. WO92/16221,

incorporated herein by reference. If a cysteine mutein is coupled to the polymer via a thiol-specific reactive group, the conjugate formed is expected to be attached to the protein at the non-native cysteine residue. During refolding of the mutein, however, the non-native cysteine might become involved in a disulfide bond and thereby free a native cysteine for PEGylation. In such cases, the polymer is attached at that native cysteine residue. Using peptide mapping, one can determine the specific PEGylation site.

Also contemplated are "dumbbell" molecules which contain 2 IGFBP-1 molecules, one at each ends of the polymer molecule.

One skilled in the art using conventional methods and following the teachings of PCT Application Publication No. WO92/16221, incorporated herein by reference, and the teachings provided herein, can readily determine the appropriate pH, concentration of protein, and ratio of protein to polymer useful for making these modified forms of IGFBPs, including IGFBP-1.

The present invention further provides a pharmaceutical composition containing the IGFBPs, including of IGFBP-1, in an acceptable pharmaceutical carrier. One carrier is physiological saline solution, but it is contemplated that other acceptable pharmaceutical carriers may also be used. In one embodiment it is envisioned that the carrier and the IGFBP-1 constitute a physiologically-compatible, slow-release formulation. The primary solvent in such a carrier may be either aqueous or non-aqueous in nature. In addition, the carrier may contain other pharmacologically-acceptable excipients for modifying or maintaining the pH, osmolarity, viscosity, clarity, color, sterility, stability, rate of dissolution, or odor of the formulation. Similarly, the carrier may contain still other pharmacologically-

acceptable excipients for modifying or maintaining the stability, rate of dissolution, release, or absorption of the IGFBP-1. Such excipients are those substances usually and customarily employed to formulate dosages for administration in either unit dose or multi-dose form.

Once the therapeutic composition has been formulated, it may be stored in sterile vials as a solution, suspension, gel, emulsion, solid, or dehydrated or lyophilized powder. Such formulations may be stored either in a ready to use form or requiring reconstitution immediately prior to administration. The preferred storage of such formulations is at temperatures at least as low as 4°C and preferably at -70°C. It is also preferred that such formulations containing IGFBP-1 are stored and administered at or near physiological pH. It is presently believed that storage and administration in a formulation at a high pH (i.e. greater than 9) or at a low pH (i.e. less than 4) is undesirable.

The pharmaceutical compositions of the present invention can be administered by intravenous, parenteral, intramuscular, subcutaneous, intraarticular injection or infusion, inhalant mists, orally active formulations, or suppositories. To achieve and maintain the desired dose of IGFBP-1, repeated doses may be administered. This method is intended to create a preselected concentration range of IGFBP-1 in the patient's blood stream. It is believed that the maintenance of circulating concentrations of IGFBP-1 of 0.1 μg to 300 μg per ml in the bloodstream may be effective in treating an IGF associated condition. The frequency of dosing will depend on pharmacokinetic parameters of the IGFBP-1 in the formulation used.

The methods of the present invention are based, in part, on experiments described in the following Examples. Briefly, the present inventors discovered that

IGFBP-1 blocks the mitogenic effects of IGF-I and IGF-II on breast cancer, colon and osteosarcoma cancer cells in vitro. Estrogen stimulates growth of breast cancers at least in part by causing the cells to secrete IGF-I or IGF-II.

Concentrations of BP-1 of 1-10 μg/ml were required to inhibit growth of the colon cancers. One of the colon carcinoma cell lines grew in serum free media, presumably because it produced its own growth factors. Growth of this cell line in serum free media was inhibited at least 50% by high concentrations of BP-1.

The present inventors have demonstrated that IGFBP-1 inhibits the mitogenic effects of IGF-I on osteosarcoma cells. An approximate 12-fold molar excess of IGFBP-1 inhibited the mitogenic effect of 50 ng/ml IGF-I by 50% on rat osteosarcoma cells.

IGFBP-1 was also shown to inhibit the proliferative response of smooth muscle cells to IGF-I. IGFBP-1 administered after angioplasty in rats significantly inhibited the intimal thickening that results from smooth muscle proliferation and extracellular matrix deposition. These results indicate that IGFBP-1 is useful in the treatment or prevention of restenosis.

In hypophysectomized rats, IGFBP-1 inhibited the growth promoting effects of IGF-I and of growth hormone. In addition, IGFBP-1, its muteins, and PEGylated IGFBP-1 inhibited IGF-I stimulation of growth of mouse 3T3 fibroblast cells.

The following examples are intended to illustrate but not limit the present invention.

Example 1

A. Purification & Refolding of IGFBP-1

E. coli cells expressing the IGFBP-1 were suspended in Buffer A (50 mM Tris, pH 7.5, 20 mM NaCl and 1 mM DTT) at a concentration of 40 ml/10 g cell paste, and were disrupted at 1800 psi using a French pressure cell. The suspension was centrifuged 20,000 x g for 30 minutes, and aliquots of the pellet & supernatant were analyzed by SDS-PAGE. A major band corresponding to the IGFBP-1 was present in the pellet, but not the supernatant. The pellet was suspended in Buffer A (40 ml/10 g cell paste), and re-centrifuged at 20,000 x g for 30 minutes. This wash procedure was repeated 2 times. The final pellet containing the IGFBP-1 was suspended in 6M guanidine, 50 mM Tris, pH 7.5, 6 mM DTT (25 ml/10 g cells) using a ground glass homogenizer. The suspension was incubated at room temperature for 15 minutes. The undissolved protein was removed by centrifugation at 20,000 x g for 30 minutes. Final concentration of the IGFBP-1 was 1.0 mg/ml. SDS-PAGE analysis of the pellet and supernatant showed that IGFBP-1 was present in the supernatant only.

The denatured and reduced IGFBP-1 was subjected to a three-step refolding procedure.

a) Oxidized glutathione, the mixed-disulfide producing reagent (GSSG), was added to the supernatant to a final concentration of 25 mM, and incubated at room temperature for 15 minutes.

b) The solution was then diluted 10 fold gradually with 50 mM tris. pH 9.7 and phenylmethylsulfonylfluoride was added to final concentration of 1mM. Final concentration of protein was 100μg/ml.

c) The refolding mixture was incubated overnight at 4°C, and then centrifuged at 20,000 x g for 15 minutes. SDS-PAGE analysis of the pellet and supernatant showed that the supernatant was composed of relatively homogeneous IGFBP-1.

Aliquots (50μl) of the supernatant were diluted to 200μl with Buffer C (0.05% TFA), injected onto a reverse phase column (RP-4, 1 x 250mm, Synchrom), and eluted with 80% acetonitrile, 0.042% TFA (Buffer D) using a linear gradient (increase of 1% Buffer D/minute) at a flow rate of 0.1 ml/minute.

A single major peak representing refolded IGFBP-1 eluted at 68 minutes. The retention time of the refolded IGFBP-1 shifted to 71.0 minutes after being completely reduced and denatured in 5 M guanidine, 50 mM Tris pH 7.5, 100 mM DTT. These results indicate that IGFBP-1 refolds to a single predominant species under the conditions described. N-terminal sequence analysis of IGFBP-1 eluting at 68.0 minutes gave the sequence Met Ala Pro Trp Gln Cys Ala Pro... (SEQ ID NO 3), which matches the N-terminal amino acid sequence of human IGFBP-1 (SEQ ID NO.: 1) except for an extra methionine residue at the N-terminus of the recombinant protein.

B. Isolation of Refolded IGFBP-1

The refold mixture (15000 ml) prepared from 590g of *E. coli* paste containing the correctly refolded IGFBP-1 was concentrated to 1800 ml, dialyzed against 20 mM sodium phosphate, pH 6.0, centrifuged at 10,000 x g for 30 minutes to remove

precipitated *E. coli* proteins and loaded onto an Q-Sepharose (Pharmacia/LKB, Piscataway, NJ) column (5.0 x 60 cm) previously equilibrated with the same buffer. The bound protein was eluted with a 5000 ml linear gradient to 0.5M NaCl at a flow rate of 20 ml/minute. 25 ml fractions were collected. A single major peak eluted at 0.3-0.4 M NaCl; 100 μl aliquots of each fraction were analyzed separately by a reverse phase chromatography column (RP-4 1 x 250 mm Synchrom). Fractions containing predominantly correctly refolded IGFBP-1 (determined from RP-4 analysis), were pooled (900 ml), the pH was adjusted to 7.5, the conductivity was adjusted to 1 mM NaCl (95 mOhm), and loaded onto a Toyopearl butyl-650 S hydrophobic interaction column (5 x 5 cm) (Supelco, Bellefonte, PA), previously equilibrated with 20 mm HEPES, pH 7.5, 1.0 M NaCl at a flow rate of 30 ml/minute.

The protein was eluted with a 1500 ml linear gradient to 20 mM HEPES, pH 7.5 at a flow rate of 40 ml/minute. A single broad peak eluted at 5 - 15% ethanol. Aliquots (10μl) of each peak fraction were analyzed by RP-4 reverse phase chromatography and SDS-PAGE. Fractions containing pure (95%) correctly refolded IGFBP-1 were pooled, concentrated to 6-8 mg/ml and assayed for bioactivity.

In all of the following experiments using IGFBP-1, the recombinant E. Coli.-expressed IGFBP-1 was used.

Example 2

IGFBP-1 inhibits the growth promoting effects of IGF-I and growth hormone in rats

Hypophysectomy (removal of the pituitary) removes the source of growth hormone from the body and results in growth cessation. Hypophysectomized animals

can be stimulated to grow by injection of exogenous growth hormone or IGF-I, as described in Schoenle et al., Nature, 296: 252-253 (1982). The effects of subcutaneously administered IGFBP-1 were tested on IGF-I and growth hormone-stimulated growth in this model. Growth was assessed by measuring body weight gain and tibial epiphyseal width.

A. IGF-I experiments

Male Sprague Dawley rats that were surgically hypophysectomized at 120-130 grams of body weight were obtained from a commercial source (Charles River, Wilmington, MA). The body weights of the rats were monitored for two to three weeks before the beginning of the experiments in order to verify completeness of the hypophysectomy. Rats gaining more than 2 grams of body weight per week were excluded from the study. Rats were injected subcutaneously twice a day at the nape of the neck for eight consecutive days with 0.2 ml of vehicle solution (40 mM HEPES, 100 mM NaCl), IGF-I (80 μg) alone, IGFBP-1 alone, or IGF-I (80 μg) combined with various molar ratios of BP-1. The molar ratios of IGFBP-1:IGF-I tested ranged from 0.04:1 to 5:1. Body weights were determined daily. The rats were sacrificed 12 hours after receiving their final injection. Their right and left tibias were removed, fixed with formalin, split at their proximal ends in a sagittal plane and stained with silver nitrate, as described in Greenspan, Endocrinology, 45:455-463 (1949). The calcified tissue was stained dark brown and the proliferating zone of cartilage appeared as a clearly defined white band. The cartilaginous epiphyseal plate was measured with a stereomicroscope equipped with a calibrated micrometer eyepiece. Approximately ten individual readings were made across each

epiphysis. The mean of the combined readings from the right and left tibias was calculated for each rat.

The results of several experiments are compiled in Table 1. Vehicle treated rats did not gain weight over the eight day test period whereas IGF-I treated rats gained an average of about six grams per rat over that time period. Rats in the 5:1 IGFBP-1:IGF-I group showed no significant weight gain, indicating that excess IGFBP-1 blocked the growth-promoting effects of IGF-I in this model. The administration of IGFBP-1 at molar ratios of 1:1 and 0.2:1 relative to IGF-I caused 50 to 75 % inhibition of IGF-I-stimulated growth. No enhancement of growth above that stimulated by IGF-I alone was measured in any group receiving IGFBP-1. In addition, the administration of IGFBP-1 alone had no significant growth promoting effects. Statistically significant inhibition of IGF-I-stimulated enlargement of the tibial epiphyseal width occurred with the administration of IGFBP-1 at molar ratios of 5:1 and 1:1 relative to IGF-I (Table 1). These data show IGFBP-1 inhibits bone and cartilage growth stimulated by IGF-I.

TABLE 1

EFFECTS OF SUBCUTANEOUSLY ADMINISTERED IGFBP-1 ON IGF-I-STIMULATED INCREASES IN BODY WEIGHT AND TIBIAL EPIPHYSEAL WIDTH MEASUREMENTS

IGF-I* (µg/ injection)	IGFBP-1* (µg/ injection)	IGFBP-1: IGF-I Molar Ratio	INCREASE IN BODY WEIGHT** (grams)	TIBIAL EPIPHYSEAL WIDTH** (mm)
0	0	0:0	0.9 ± 0.8	0.124 ± 0.003
80	0	0:1	5.6 ± 0.8	0.175 ± 0.006
80	9.6	0.04:1	4.7 ± 0.5	0.165 ± 0.010
80	48	0.2:1	1.8 ± 0.8	0.181 ± 0.007
80	240	1:1	2.6 ± 0.9	0.144 ± 0.008
80	1200	5:1	1.4 ± 0.3	0.133 ± 0.006
0	9.6	0.04:0	-2.6 ± 0.6	0.126 ± 0.007
0	48	0.2:0	-0.5 ± 0.5	0.136 ± 0.005
0	240	1:0	-0.25 ± 0.6	0.132 ± 0.005
0	1200	5:0	-1.3 ± 0.4	0.124 ± 0.003

* Rats received two injections per day.

** Values are means ± standard errors of the mean for 8 to 15 rats per group.

B. Growth hormone experiments

The design of this experiment was the same as the IGF-I experiments except that rats received injections of growth hormone rather than IGF-I. Growth hormone and IGFBP-1 were injected subcutaneously at separate injection sites. IGFBP-1 was administered twice a day at 10 mg/kg per injection, a dose that was equivalent to that administered in the 5:1 molar excess ratio in the experiments described above. Human pituitary-derived growth hormone (Sigma Chemical Company, St. Louis,

MO.) was administered twice daily at 15 mU per injection. This dose of growth hormone stimulated a stronger growth response in the rats than did the dose of IGF-I used in the previous experiment. The growth hormone-treated rats gained 12 grams per rat on average during the six day administration period (Table 2). Weight gain was inhibited by about 75% in the IGFBP-1-treated rats. Growth hormone stimulated an approximate two-fold increase in tibial epiphyseal width relative to vehicle-treated animals (Table 2). The growth hormone-stimulated increase in tibial epiphyseal width was inhibited by about 75 % by co-administration of IGFBP-1 (Table 2).

TABLE 2

EFFECTS OF SUBCUTANEOUSLY ADMINISTERED IGFBP-1 ON GROWTH HORMONE-STIMULATED INCREASES IN BODY WEIGHT AND TIBIAL EPIPHYSEAL WIDTH MEASUREMENTS

GROWTH HORMONE* (per injection)	IGFBP-1* (µg/injection)	INCREASE IN BODY WEIGHT** (grams)	TIBIAL EPIPHYSEAL WIDTH** (mm)
0	0	-1.2 ± 0.9	0.154 ± 0.009
15 mU	0	12 ± 1.3	0.314 ± 0.014
15 mU	1200	3.4 ± 1.1	0.195 ± 0.010

* Rats received two injections per day.

** Values are means ± standard errors of the means for 5 rats per group.

The above experiments indicate that IGFBP-1 is capable of inhibiting the growth promoting effects of both IGF-I and growth hormone in rats.

Example 3

IGFBP-1 inhibits growth of a human breast carcinoma cell line in vitro

The biological effects of IGF-I, IGF-II and IGFBP-1 were determined on a human breast cancer cell line. The human breast carcinoma cell line, MCF7, was obtained from the American Type Culture Collection located in Rockville,MD (catalogue number HTB 22). The cells were maintained in Eagle's minimal essential medium (available from Mediatech, Herndon,VA) containing 10% fetal bovine serum, 10 μg/ml insulin, 2 mM glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin and nonessential amino acids (Irvine Scientific). For cell proliferation assays, the MCF7 cells were detached from plates by brief treatment with trypsin and EDTA. Cells were plated in 96 well tissue culture plates (Costar Corporation, Cambridge, MA) at 2 x 10^4 per well in a serum free medium (Eagle's minimal essential medium containing 1 mg/ml bovine serum albumin, 2 mM glutamine, 1 mM sodium pyruvate, 100 Units/ml penicillin, 100 μg/ml streptomycin and non-essential amino acids). Varying dilutions of IGF-I, IGF-II or IGFBP-1 were added to the wells in a final volume of 200 μl. After 4 days at 37°C, 20 μl of a 5 mg/ml solution of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; available from Sigma Chemical Company, catalogue # M5655) was added to each well. The cells were incubated for an additional 6 hours at 37°C. The cells and the hydrolyzed MTT were solubilized by the addition of 50 μl of a solution of 50% dimethyl formamide, 20% sodium dodecyl sulfate, pH 4.7. After overnight incubation at 37°C, the hydrolyzed MTT was quantitated by measuring the optical density of the liquid at 570 nm and subtracting the 650 nm optical density background

with a VMAX kinetic microplate reader (Molecular Devices Corporation, Palo Alto,CA).

Both IGF-I and IGF-II caused proliferation of MCF7 cells, as evidenced by an increase in the optical densities of the cell cultures (Table 3). IGF-I was about 5 times more potent than IGF-II at stimulating proliferation of MCF7 cells. MCF7 cell proliferation occurred at IGF-I concentrations ranging from about 1 to 120 ng/ml and at IGF-II concentrations ranging from about 10 to 1200 ng/ml. IGFBP-1 inhibited IGF-I and IGF-II stimulated proliferation of MCF7 cells in a dose dependent manner (Tables 4 and 5). This was determined by incubating MCF7 cells with 60 ng/ml IGF-I, or 300 ng/ml IGF-II, in the presence of increasing amounts of IGFBP-1. The tissue culture medium used was the same as that used to maintain the cells except that it did not contain serum or insulin.

For IGF-I, the IGFBP-1 concentrations tested ranged from 6 to 13,600 ng/ml. Approximately 50% growth inhibition occurred at a IGFBP-1 concentration of about 180 ng/ml, which corresponds to about a 1:1 molar ratio of IGF-I:IGFBP-1 (Table 4). Essentially complete growth inhibition occurred at IGFBP-1 concentrations exceeding 4000 ng/ml, which corresponds to an approximate 20-fold molar excess of IGFBP-1.

The IGFBP-1 concentrations tested for IGF-II ranged from about 30 ng/ml to about 23,000 ng/ml. Approximately 50% inhibition of the IGF-II growth response occurred at a IGFBP-1 concentration of about 840 ng/ml, which is slightly greater than a 1:1 molar ratio of IGF-I:IGFBP-1 (Table 5). Essentially complete growth inhibition occurred at IGFBP-1 concentrations exceeding 22,000 ng/ml, which corresponds to slightly greater than a 20-fold molar excess of IGFBP-1.

TABLE 3

STIMULATION OF MCF-7 BREAST CANCER CELL GROWTH BY IGF-I AND IGF-II

IGF-I* (ng/ml)	O.D.** 570-650 nm	IGF-II* (ng/ml)	O.D.** 570-650 nm
0	0.121	0	0.121
1	0.140	10	0.139
2	0.140	19	0.149
4	0.150	38	0.181
8	0.169	75	0.195
15	0.189	150	0.216
30	0.217	300	0.256
60	0.273	600	0.321
120	0.330	1200	0.411

* IGF-I and IGF-II concentrations rounded to the nearest whole number.

** Optical density at 570 nm minus optical density at 650 nm. Means of triplicate wells. Standard deviations were less than 11% of the means.

TABLE 4

INHIBITION OF IGF-I-STIMULATED MCF-7
BREAST CANCER CELL GROWTH BY IGFBP-1

IGFBP-1* (ng/ml)	IGF-I (ng/ml)	O.D.** 570-650 nm	% INHIBITION
0	0	0.260	
0	60	0.610	0%
6	60	0.543	19%
19	60	0.472	39%
56	60	0.489	35%
168	60	0.443	48%
504	60	0.372	68%
1,511	60	0.330	80%
4,533	60	0.286	93%
13,600	60	0.242	105%

* IGFBP-1 concentrations rounded to the nearest whole number.

** Optical density at 570 nm minus optical density at 650 nm. Means of triplicate wells. Standard deviations were less than 8% of the means.

TABLE 5

INHIBITION OF IGF-II STIMULATED MCF-7
BREAST CANCER CELL GROWTH BY IGFBP-1

IGFBP-1 (ng/ml)	IGF-II (ng/ml)	O.D. * 570-650 nm	% INHIBITION
0	0	0.126	
0	300	0.357	0%
31	300	0.345	5%
93	300	0.299	25%
280	300	0.277	35%
839	300	0.243	49%
2,519	300	0.181	76%
7,556	300	0.161	85%
22,667	300	0.144	92%

* Optical density at 570 nm minus optical density at 650 nm. Means of triplicate wells. Standard deviations were less than 11% of the means.

Example 4

IGFBP-1 inhibits growth of human colon carcinoma cell lines in vitro

The biological effects of IGF-I and IGFBP-1 were tested on a number of human colon cancer cell lines. Six human colon carcinoma cell lines were obtained from the American Type Culture Collection located in Rockville, MD. These cell lines were SK-CO-1 (HTB 39), LS 174T (CL 188), DLD-1 (CCl 221), HT-29 (HTB 38), COLO-205 (CCL 222) and Caco-2 (HTB 37). The designations within parentheses refer to the ATCC catalog number. These cell lines were selected

because they all form tumors in nude mice according to the descriptions provided in the American Type Culture Collection catalogue. The cells were maintained in Eagle's Minimal Essential Medium (Mediatech, Herndon, VA) containing 10% fetal bovine serum, 2 mM glutamine, 100 Units/ml penicillin and 100 ug/ml streptomycin.

The effect of IGF-I on these six colon cancer cell lines was determined as follows. When the cells reached 90-100% confluency, the cells were detached from the plates by treating them briefly with a trypsin/EDTA solution. The cells were washed several times, counted and resuspended in serum-free media (Eagle's Minimal Essential Medium containing 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin) at a concentration of $1x10^5$/ml. 100 μl of the cell suspension was added per well of a 96 well tissue culture plate (Corning Glass Works, Rochester, NY). 100 μl of serum-free media containing varying amounts of IGF-I were added to the wells and the cultures mixed gently by pipetting. The plates were incubated at 37°C for 3 days. At this time, cell number was quantitated using a crystal violet dye assay. Media was suctioned off the cells and 150 μl of crystal violet stain [2g of crystal violet (Aldrich Chemical Company,Inc., Milwaukee, WI) dissolved in a solution containing 270 ml 37% formaldehyde and 20 ml of potassium phosphate pH 7.0] was added per well. Twenty minutes later the liquid was suctioned off and the wells washed 3 times with phosphate buffered saline. 200 μl of extraction buffer (50% ethanol, 0.1 M sodium citrate pH 4.2) was added per well and the plates left overnight at room temperature. The optical density of the wells at 570 nm was determined the next day using a microplate reader (Molecular Devices, Palo Alto, CA).

All six cell lines proliferated in response to IGF-I (Tables 6 and 7), as evidenced by an increase in the optical densities of the cell cultures. The Caco-2 cell line grows well in serum-free media, suggesting that it produces one or more endogenous growth factors. Growth of the Caco-2 cell line in serum-free media was enhanced by IGF-I (Table 6). The other colon cancer cell lines examined did not show significant cell proliferation in serum-free media under the conditions tested. However, they all proliferated in response to IGF-I as evidenced by an increase in the optical densities of the cultures (Tables 6 and 7).

TABLE 6

STIMULATION OF GROWTH OF HUMAN COLON CANCER CELL LINES BY IGF-I

IGF-I* (ng/ml)	O.D.** 570 nm				
	SK-CO-1	CaCO-2	DLD-1	COLO-205	LS 174T
0	0.156	1.253	0.474	0.138	0.144
1	0.174	1.402	0.493	0.154	0.165
4	0.218	1.472	0.497	0.163	0.195
16	0.337	1.796	0.532	0.178	0.242
63	0.368	1.923	0.558	0.189	0.236
250	0.391	2.022	0.634	0.219	0.225
1000	0.376	1.943	0.721	0.218	0.212

* IGF-I concentrations rounded to the nearest whole number.

** Optical density at 570 nm. Means of triplicate wells.

TABLE 7

STIMULATION OF GROWTH OF HUMAN HT-29 COLON CANCER CELLS BY IGF-I

IGF-I * (ng/ml)	O.D.** 570 nm
0	0.434
1	0.557
3	0.627
13	0.694
53	0.665
213	0.631
850	0.627

* IGF-I concentrations rounded to the nearest whole number.

** Optical density at 570 nm. Means of triplicate wells.

The effect of IGFBP-1 on growth of these cell lines was then determined. Rather than determine the effect of IGFBP-1 on IGF-I-stimulated growth of the cells, it was determined whether IGFBP-1 could inhibit growth of the cells in the presence of serum, which may be more representative of the in vivo situation. Cells were detached from plates by brief trypsin/EDTA treatment, washed, resuspended in Eagle's Minimal Essential Medium containing 4% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin at a concentration of 1 x 10^5/ml and 100 μl of the cell suspension added per well of a 96 well tissue culture plate. IGFBP-1 was diluted to varying concentrations in serum-free Eagle's Minimal Essential media containing 2 mM glutamine, 100

units/ml penicillin and 100 μg/ml streptomycin and 100 μl of the mixture added per well of the 96 well plate. The cell cultures were mixed by gentle pipetting and incubated for 3 days at 37°C. The final serum concentration was 2%. Cell number was quantitated at this time using the crystal violet dye assay described above. IGFBP-1 caused significant inhibition of the growth response of four of the cell lines (Caco-2, COLO-205, HT-29 and SK-CO-1) in the presence of 2% serum (Tables 8 and 9). Little growth inhibition was seen until IGFBP-1 levels reached several hundred ng/ml. The maximum growth inhibition observed was between 30% and 100% (at IGFBP-1 levels of 10-20 μg/ml). IGFBP-1 had little effect on serum-stimulated growth of the LS 174T and DLD-1 cell lines (maximum inhibition of 9 and 22%, respectively, at 10-20 ug/ml IGFBP-1). The concentrations of free IGF-I and IGF-II in the serum were not determined.

TABLE 8

INHIBITION OF SERUM-STIMULATED GROWTH OF HUMAN COLON CANCER CELL LINES BY IGFBP-1

		CaCO-2		COLO-205		HT-29	
SERUM (%)	IGFBP-1* (ng/ml)	O.D.** 570 nm	% IN-HIBITION	O.D.** 570 nm	% IN-HIBITION	O.D.** 570 nm	% IN-HIBITION
0	0	1.253		0.138		0.145	
2	0	1.999	0%	0.574	0%	0.556	0%
2	3	2.076	-10%	0.591	- 4%	0.510	11%
2	16	2.023	- 3%	0.542	7%	0.483	18%
2	80	1.926	10%	0.581	- 2%	0.497	14%
2	400	1.741	35%	0.466	25%	0.407	36%
2	2,000	1.526	63%	0.386	43%	0.344	52%
2	10,000	1.266	98%	0.304	62%	0.300	62%

* IGFBP-1 concentrations rounded to the nearest whole number.

** Optical density at 570 nm. Means of triplicate wells.

TABLE 9

INHIBITION OF SERUM-STIMULATED GROWTH OF HUMAN SK-CO-1 COLON CANCER CELLS BY IGFBP-1

SERUM (%)	IGFBP-1* (ng/ml)	SK-CO-1	
		O.D.** 570 nm	% INHIBITION
0	0	0.156	
2	0	0.507	0%
2	1	0.519	-3%
2	6	0.514	-2%
2	32	0.496	3%
2	160	0.432	21%
2	800	0.406	29%
2	4,000	0.395	32%
2	20,000	0.386	34%

* IGFBP-1 concentrations rounded to the nearest whole number.

** Optical density at 570 nm. Means of triplicate wells.

Example 5

IGFBP-1 inhibits growth of a human osteosarcoma cell line in vitro

The ability of BP-1 to inhibit IGF-I stimulated growth of an osteosarcoma was determined using a rat osteosarcoma cell line, UMR-106 (CRL 1661), obtained from the American Type Culture Collection (Rockville, MD). The cells were maintained in Ham's F12 medium (available from Mediatech, Herndon, VA) containing 7% fetal bovine serum, 100 Units/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine. The cells proliferate in response to IGF-I.

well of a 48 well tissue culture plate (Costar Corporation, Cambridge, MA). When the cells became confluent (after approximately 3 days at 37°C), the cells were washed twice with phosphate buffered saline (PBS) and pre-incubated for 24 hours in the above medium lacking fetal bovine serum. After the pre-incubation, the medium was removed and replaced by 0.5 ml of serum-free Ham's F12 medium containing serial dilutions of IGF-I (1 to 1,000 ng/ml). The plates were incubated for an additional 20-24 hours at 37°C. Each well was then pulsed with 0.5 μCi of ^{3}H-thymidine (NEN research products, Dupont Co., Boston,NA) for 4 hours at 37°C, then washed three times with cold PBS. DNA was precipitated by adding cold 7% trichloroacetic acid (J.T.Baker Inc., Phillipsburg,NJ) to the cells. After rinsing with 95% ethanol, the cells were solubilized by the addition of 0.3 M NaOH. Aliquots were removed and counted in a scintillation counter to quantitate the amount of ^{3}H-thymidine incorporated into DNA. All assays were performed in triplicate.

As shown in Table 10, IGF-I caused a dose-dependent increase in ^{3}H-thymidine incorporated into DNA. The maximal response was about six times over the level seen in the absence of IGF-I. The ED_{50} for IGF-I ranged from 4-20 ng/ ml in different experiments.

TABLE 10

STIMULATION OF RAT OSTEOSARCOMA UMR-106

CELL GROWTH BY IGF-I

IGF-I* (ng/ml)	cpms**
0	34,624
1	40,636
2	54,814
4	99,715
8	145,525
16	141,274
31	187,174
63	189,257
125	194,254
250	184,857
500	212,929
1000	197,268

* IGF-I concentrations rounded to the nearest whole number.

** Counts per minute. Means of triplicate wells.

The effect of IGFBP-1 on IGF-I stimulated growth of UMR-106 cells was determined using the above assay except for the following changes. After the pre-incubation step the cells were incubated with serum-free Ham's F12 medium containing 50 ng/ml IGF-I and varying concentrations of BP-1 (200 to 16,000 ng/ml). The medium also contained the 2 mM glutamine, 100 Units/ml penicillin and 100 μg/ml streptomycin. After 20-24 hours at 37°C, the cells were pulsed with 0.5 μCi of ^{3}H-thymidine for 4 hours, rinsed three times with cold PBS, and DNA precipitated with cold 7% trichloroacetic acid. The cells were rinsed with 95% ethanol, solubilized in 0.3 M NaOH and aliquots counted in a scintillation counter.

The results of one of these experiments are shown in Table 11. The data indicate that IGFBP-1 inhibits the mitogenic effect of IGF-I on osteosarcoma cells. An approximate 12-fold molar excess of IGFBP-1 (2,000 ng/ml) inhibited the mitogenic effect of 50 ng/ml IGF-I by 50%. Essentially complete inhibition of the mitogenic effect of 50 ng/ml IGF-I was seen with 50-100 fold molar excess of IGFBP-1 (8,000 -16,000 ng/ml). The amount of IGFBP-1 required to inhibit effects of IGF-I in these experiments is greater than that observed in other experiments and with other cell lines. This probably is due to the fact that 50 ng/ml IGF-I gave a maximal mitogenic response in this experiment.

TABLE 11

INHIBITION OF IGF-I-STIMULATED GROWTH OF RAT OSTEOSARCOMA UMR-106 CELLS BY IGFBP-1

IGF-I (ng/ml)	IGFBP-1 (ng/ml)	cpms*	% INHIBITION
0	0	32,145	
50	0	188,933	0%
50	200	172,513	10%
50	400	173,234	10%
50	800	157,475	20%
50	2,000	112,265	49%
50	4,000	78,041	71%
50	8,000	51,470	88%
50	16,000	34,448	99%

* Counts per minute. Means of triplicate wells.

Example 6

IGFBP-1 inhibits growth of smooth muscle cells

IGFBP-1 was tested to determine if it could inhibit the proliferative response of smooth muscle cells to IGF-I. A rat smooth muscle cell-like cell line, A10, was obtained from the American Type Culture Collection located in Rockville, MD (catalogue # CRL 1476). The A10 cell line has been characterized by B.W. Kimes and B.L. Brandt, Experimental Cell Research, 98:349-366 (1976). The cells were maintained in DMEM medium (Dulbecco's Modification of Eagle's Medium, available from Mediatech, Inc. Herndon, VA) containing 10% fetal bovine serum, 2 mM glutamine, 100 Units/ml penicillin and 100 μg/ml streptomycin. For proliferation assays the cells were detached from plates by brief treatment with a trypsin/EDTA solution, washed once with serum containing medium, twice with serum-free medium and counted using a hemocytometer. The cells were resuspended at a concentration of 2×10^5/ml in

serum-free DMEM medium containing 2 mM glutamine, 100 Units/ml penicillin and 100 μg/ml streptomycin. 100 μl of the cell suspension was aliquoted per well of a 96 well tissue culture plate (Corning Glass Works, Rochester, NY). 100 μl of serum-free medium containing increasing amounts of IGF-I (0, 2, 20, 200, or 2000 ng/ml) were added to appropriate wells and the plates incubated for 3 days at 37°C. At this time cell number was quantitated using the crystal violet dye assay described in Example 3.

IGF-I caused a dose-dependent increase in cell number, as measured by an increase in the optical density of the wells (Table 12). The maximum proliferative response occurred at an IGF-I concentration of 100 ng/ml.

TABLE 12

STIMULATION OF RAT A-10 SMOOTH MUSCLE CELL GROWTH BY IGF-I

IGF-I (ng/ml)	O.D.* 570 nm
0	0.318
1	0.317
10	0.451
100	0.577
1000	0.536

* Optical density at 570 nm. Means of 6 wells.

The effect of recombinant IGFBP-1 on IGF-I-stimulated growth of A-10 cells was determined using the above cell proliferation assay. The assay was performed in an identical manner except that the test wells contained 100 ng/ml IGF-I. Some wells also contained IGFBP-1 at concentrations ranging from 1-10,000 ng/ml.

IGFBP-1 caused a dose-dependent decrease in cell number as evidenced by a decrease in the optical densities of the cell cultures (Table 13). At a

concentration of 1000 ng/ml, IGFBP-1 reduced the cell number to that seen without any exogenous IGF-I (referred to as baseline proliferation). At a concentration of 10 μg/ml, IGFBP-1 reduced the cell number to below that seen in serum-free media, suggesting that rat A-10 cells produce endogenous IGF-I or IGF-II. These data also indicate that IGFBP-1 inhibits the proliferative response of rat smooth muscle cells to IGF-I.

TABLE 13

INHIBITION OF IGF-I-STIMULATED GROWTH OF RAT A-10 SMOOTH MUSCLE CELLS BY IGFBP-1

		EXPERIMENT 1		EXPERIMENT 2	
IGFBP-1 (ng/ml)	IGF-I (ng/ml)	O.D.* 570 nm	% INHIBITION	O.D.* 570 nm	% INHIBITION
0	0	0.192		0.230	
0	100	0.290	0	0.353	0
10	100	0.253	38%	0.334	19%
100	100	0.249	42%	0.282	59%
1,000	100	0.197	95%	0.236	95%
10,000	100	0.157	136%	0.183	137%

* Optical density of 570 nm. Means of triplicate wells.

Example 7

Preparation of IGFBP-1 muteins

A. Construction of IGFBP-1 muteins

Two IGFBP-1 muteins, C98 and C101, were constructed by mutagenesis of the IGFBP-1 DNA sequence contained in plasmid pJU1021, ATCC Accession No. 67730. In the C98 mutein, the serine at position 98 of the mature protein sequence has been changed to a cysteine residue. In the C101 mutein, the serine at position 101 of the mature protein sequence has been changed to a cysteine

residue. Residue numbering is based upon SEQ ID No.:1. Mutagenesis was done utilizing the polymerase chain reaction (PCR) technique.

The C98 mutein was made using plasmid T88IQ:IGFBP-1 DNA as the starting template DNA. Plasmid pT88:IGFBP-1 contains the wild type IGFBP-1 coding sequence in plasmid pT88IQ.

The expression vector pT88IQ is a derivative of the expression vector pT3XI-2. The vector pT3XI-2 was constructed in the following manner. The starting plasmid for this construction was plasmid pKK223-3 purchased from Pharmacia. Plasmid pKK223-3 carries a partial gene for tetracycline resistance. This nonfunctional gene was replaced by a complete tetracycline resistance gene carried on plasmid pBR322. Plasmid pKK223-3 was digested completely with SphI and partially with BamH1. A 4.4 kilobase pair fragment was gel purified and combined with a synthetic adapter (SEQ ID NO:4):

5' GATCTAGAATTGTCATGTTTGACAGCTTATCAT 3'
3' ATCTTAACAGTACAAACTGTCGAATAGTAGC 5'
BglII ClaI

and a 539 basepair fragment of DNA from a ClaI, SphI digest of the tetracycline resistance gene of pBR322 (PL Biochemicals, 27-4891-01). The resulting plasmid was designated pCJ1.

Next, a XhoI linker purchased from New England Biolabs (Beverly, Massachusetts) was inserted into plasmid pCJ1's PvuII site to form plasmid pCJX-1. This insertion disrupts the rop gene which controls plasmid copy number. Next, an EcoRI fragment containing the lacI gene was purified from plasmid pMC9 (Calos et al., 1983), then inserted into the XhoI site with XhoI to EcoRI adapters. The polylinker region in plasmid pKK223-3 was next replaced with a polylinker containing additional sites by cutting with EcoRI and PstI (SEQ ID NO:5):

5' AATTCCCGGG TACCAGATCT GAGCTCACTA
GTCTGCA 3'
3' GGGCCC ATGGTCTAGA CTCGAGTGAT CAG 5'

The plasmid vector so obtained is designated pCJXI-1.

Finally, the tetracycline resistance gene was replaced with a similar gene which had the recognition sites for restriction enzymes HindIII, BamH1, and SalI destroyed by bisulfite mutagenesis. The following procedure was used to mutate the tetracycline resistance gene of pBR322. Plasmid pBR322 was cut with HindIII, then mutagenized with sodium bisulfite (Shortle and Botstein, 1983). The mutagenized DNA was ligated to form circular DNA, then cut with HindIII to linearize any plasmid that escaped mutagenesis. This digestion mixture was used to transform *E. coli* JM109 (Yanisch-Perron et al., 1985). Tetracycline-resistant colonies were isolated and checked for loss of the HindIII site in the tetracycline resistance gene of the plasmid. A successfully mutated plasmid was designated pT1. A similar procedure was followed to mutagenize the BamH1 site in pT1, yielding plasmid pT2. Plasmid pT2 in turn was mutagenized to remove the SalI site, forming plasmid pT3. A ClaI-StyI fragment of pT3 carrying the mutated tetracycline resistance gene was isolated and used to replace the homologous fragment of pCJXI-1 to form pT3XI-2. The mutated tetracycline resistance gene still encodes for a functional protein. Downstream of the tac promoter region, a polylinker was introduced which contains, among other sites, BamH1 and KpnI restriction sites useful for cloning genes for expression in *E. coli* as described below.

As in pT3XI-2, the expression of the cloned gene containing the pT88IQ vector is driven by the tac promoter. Translation starts at the ATG of the unique NdeI recognition sequence CATATG (a downstream NdeI site was eliminated so that this start site NdeI sequence would be unique). There is a polylinker downstream of the NdeI site to facilitate insertion of the desired gene. In addition, the XhoI fragment containing the lacI region is replaced by a truncated fragment which eliminates the lacZ promoter and the operator region which is a binding site for the lac repressor. The lacI region in the replacement also carries the lacIq mutation -- a single base substitution which results in an increase in lac repressor production (Muller-Hill et al., Proc. Nat'l Acad. Sci. (U.S.A.) 59:1259-1264 (1968)).

The specific differences between pT3XI-2 and pT88IQ are as follows:

1. The cloning site region.

Between the EcoRI site upstream of the polylinker and the HindIII site at the downstream end of the polylinker, the following 135-mer sequence was substituted (SEQ ID NO:6):

5'
>CACAACGGTTTCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGA
TATACATATGGCTAGCATGACTGGTGGACAGCAAATGGGTCGGGATCCC
GGGTACCGTCGACGAGCTCTTCGAACTAGTCCGCGGT > 3'

This sequence contains an NdeI site (underlined) at the start codon for expression and a polylinker containing recognition sites for BamHI, XmaI, KpnI, SalI, SacI, BstBI, SpeI and SacII.

2. The downstream NdeI site.

There is an NdeI site in pT3XI-2 about 2.4 Kb downstream of the cloning region. This site was eliminated so that the NdeI site at the start codon as described above was unique in pT88IQ. The site was changed from 5' > CATATG > 3' to 5' > CATATATG > 3', eliminating the NdeI recognition sequence.

3. The lacIq region.

The region in pT3XI-2 between the two XhoI sites containing the lacI region was replaced by the 1230 base sequence shown below:

lacIq sequence of pT88IQ (1230 BP) (SEQ ID NO:7)

CCATGGCTGG TGCCTAATGA GTGAGCTAAC TCACATTAAT
TGCGTTGCGCTCACTGCCCG CTTTCCAGTC GGGAAACCTG
TCGTGCCAGC TGCATTAATG AATCGGCCAA CGCGCGGGGA
GAGGCGGTTT GCGTATTGGG CGCCAGGGTG GTTTTTCTTT
TCACCAGTGA GACGGGCAAC AGCTGATTGC CCTTCACCGC
CTGGCCCTGA GAGAGTTGCA GCAAGCGGTC CACGCTGGTT
TGCCCCAGCA GGCGAAAATC CTGTTTGATG GTGGTTGACG
GCGGGATATA ACATGAGCTG TCTTCGGTAT CGTCGTATCC

CACTACCGAG ATATCCGCAC CAACGCGCAG CCCGGACTCG
GTAATGGCGC GCATTGCGCC CAGCGCCATC TGATCGTTGG
CAACCAGCAT CGCAGTGGGA ACGATGCCCT CATTCAGCAT
TTGCATGGTT TGTTGAAAAC CGGACATGGC ACTCCAGTCG
CCTTCCCGTT CCGCTATCGG CTGAATTTGA TTGCGAGTGA
GATATTTATG CCAGCCAGCC AGACGCAGAC GCGCCGAGAC
AGAACTTAAT GGGCCCGCTA ACAGCGCGAT TTGCTGGTGA
CCCAATGCGA CCAGATGCTC CACGCCCAGT CGCGTACCGT
CTTCATGGGA GAAAATAATA CTGTTGATGG GTGTCTGGTC
AGAGACATCA AGAAATAACG CCGGAACATT AGTGCAGGCA
GCTTCCACAG CAATGGCATC CTGGTCATCC AGCGGATAGT
TAATGATCAG CCCACTGACG CGTTGCGCGA GAAGATTGTG
CACCGCCGCT TTACAGGCTT CGACGCCGCT TCGTTCTACC
ATCGACACCA CCACGCTGGC ACCCAGTTGA TCGGCGCGAG
ATTTAATCGC CGCGACAATT TGCGACGGCG CGTGCAGGGC
CAGACTGGAG GTGGCAACGC CAATCAGCAA CGACTGTTTG
CCCGCCAGTT GTTGTGCCAC GCGGTTGGGA ATGTAATTCA
GCTCCGCCAT CGCCGCTTCC ACTTTTTCCC GCGTTTTCGC
AGAAACGTGG CTGGCCTGGT TCACCACGCG GGAAACGGTC
TGATAAGAGA CACCGGCATA CTCTGCGACA TCGTATAACG
TTACTGGTTT CACATTCACC ACCCTGAATT GACTCTCTTC
CGGGCGCTAT CATGCCATAC CGCGAAAGGT TTTGCACCAT
TCGATGGTGT CGGAATTAAT TCAGCCATGG

This substituted region eliminates the lacZ promoter and the operator region which is a binding site for the lac repressor. It also contains the lacIq mutation which causes an increase in lac repressor synthesis (Muller-Hill et al., supra.).

The IGFBP-1 DNA sequence was isolated from plasmid pJU1021 by digestion with the restriction enzymes Xba I and HindIII and purified by agarose gel electrophoresis using NA-45 paper (Schleicher and Schuell, Keene, NH), following the manufacturer's instructions. The isolated IGFBP-1 DNA fragment was cloned into plasmid pT88IQ that had been digested with XbaI and HindIII and

gel-purified as above. A correctly reconstructed plasmid was named pT88IQ:IGFBP-1. The 5' oligonucleotide primer (IGFBP-1-5') used in the PCR mutagenesis reaction has the sequence 5' CCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGA 3' (SEQ ID NO.: 8). The 3' oligonucleotide primer (IGFBP-1-C98) has the sequence 5' CAGGAGCTCCTCCTCAGTTATCTCCGTGCTCTCTGGGCATTCAGGGCTCC CTGCCTCTGCAGCATGGGG 3' (SEQ ID NO.: 9). The PCR was performed in a 50 μl reaction mixture containing 10 mM Tris-HCl pH 8.3, 50 mM KCL, 2.5 mM $MgCl_2$, 0.001% gelatin, 500 μM each of dATP, dCTP, dGTP and TTP, 30 picomoles each of the IGFBP-1-5' and IGFBP-1-C98 primers, 1-10 ng pT88IQ:IGFBP-1 plasmid DNA and 5 Units "AmpliTaq" Taq DNA polymerase (Perkin-Elmer Cetus, marketed by Roche Molecular Systems, Inc., Branchburg, NJ). The PCR conditions were an initial 3 min incubation at 96°C, 35 cycles of (96°C for 1 min, 66°C for 1 min, 72°C for 1.5 min) and a final 10 min incubation at 72°C.

The C101 mutein was made using an agarose gel-purified DNA fragment containing the wild type IGFBP-1 coding sequence as the starting template DNA. The IGFBP-1 coding sequence was obtained by digesting plasmid pJU1020 with NdeI and HindIII and purifying the approximate 0.8 kb IGFBP-1-coding DNA fragment by agarose gel electrophoresis. The 5' oligonucleotide primer used in the PCR mutagenesis reaction was the same as was used to construct the S98C mutein (IGFBP-1-5'). The 3' oligonucleotide primer (IGFBP-1-C101) has the sequence 5' CCCGAGCTCCTCCTCAGTTATCTCCGTGCACTCTGGGCTTTCAGGGCTCC CTGC 3' (SEQ ID NO.: 10) The PCR was performed in a 100 ul reaction mixture containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 200 uM each of dATP, dCTP, dGTP and TTP, 20 picomoles of the IGFBP-1-5' and IGFBP-1-C101 primers and 2.5 Units of "AmpliTaq" Taq DNA polymerase. The PCR conditions were 30 cycles of (95°C for 1 min, 50°C for 1 min, 72°C for 1 min) followed by a 10 min incubation at 72°C.

After the PCR, the reaction mixtures were passed through ChromaSpin 100 columns (ClonTech,Palo Alto,CA, catalogue number K1332-2) to remove

nucleotides and unincorporated DNA primers. The DNA fragments were digested with XbaI and SacI and bands of the correct size (approximately 0.43 kb) purified by agarose gel electrophoresis as described above. The purified DNA fragments were ligated to XbaI + SacI digested pT88IQ:IGFBP-1 plasmid DNA. The ligation mixtures were used to transform E. coli strain DH5 alpha (available form ClonTech Laboratories, Inc., Palo Alto, CA) and plated on LB agar plates containing 50 ug/ml ampicillin. Plasmid DNAs were prepared from several colonies resulting from each transformation and sequenced on both strands across the inserted region. A plasmid with the correct sequence was selected for each mutein. They are named clones C101-3 (C101 mutein) and C98-12 (C98 mutein).

The mutated IGFBP-1 genes were then transferred back into plasmid pT5T (Eisenberg, S.P. et al., Nature, 343: 341-346 (1990). This was done by digesting plasmid DNA from clones C101-3 and C98-12 with NdeI and HindIII, gel-purifying the approximate 800 bp band containing the mutant IGFBP-1 gene, and ligating them to pT5T plasmid DNA that had been digested with the same restriction enzymes. The ligation mixtures were used to transform E. coli strain BL21/DE3 and plated on LB agar plates containing 50 ug/ml ampicillin. Plasmid DNAs were prepared from several colonies resulting from each transformation. Clones with the correct sequences were named pT5T:IGFBP-1-C98 (C98 mutein) and pT5T:IGFBP-1-C101 (C101 mutein).

B. Preparation of Washed Inclusion Bodies

E. Coli cells expressing either the C98 or C101 mutein were grown in a 10 liter fermenter. Cells obtained from the fermenter were resuspended in breaking buffer (50 mM Tris, 25 mM NaCl, 1 mM Dithiothreitol ("DTT"), pH 7.5) at a ratio of 6ml buffer / g cells. The cells were disrupted at 10,000 PSI using a French pressure cell. The suspension was centrifuged at 17,700 x g for 30 minutes. The pellet, which contains the inclusion bodies, was washed by resuspension in the breaking buffer and recentrifuged at 17,700 x g. The washed and recentrifuged pellet can be stored frozen until processing. Approximately 80% of the protein contained in the pellet was mutein.

C. Mutein Refolding

Each mutein was first denatured by solubilizing the pellet in 6M Guanidine HCl, 50 mM Tris, 6mM DTT, pH 7.5 (1g pellet for every 10 ml buffer) using a cell homogenizer.

Refolding was initiated by adding oxidized glutathione ("GSSG") to the solubilized pellet to a final concentration of 23 mM. The solution was incubated at room temperature for 15 minutes and then diluted gradually with 50 mM Tris, pH 9.7 to a final protein concentration of 100 μg/ml and a final guanidine concentration of 0.6M. The protein concentration was determined by the Coomassie Blue protein assay. (Pierce, Rockford, IL). Cysteine and phenyl-methanesulfonyl fluoride were then added to final concentrations of 5.6mM and 1mM, respectively. The refold solution was incubated at 4°C overnight.

Refolding was monitored by analyzing a 100 μl aliquot of the refold solution on a C4 reverse phase column (RP-4 1X250mm, Synchrom, Lafayette, In). The C4 column was equilibrated with 2% acetonitrile (CH_3CN), 0.05% trifluoracetic acid ("TFA"). The 100μl aliquot of the refold solution was injected onto the equilibrated column and eluted using a flow rate of 0.25 ml/min with a linear gradient to 60% CH_3CN, 0.05% TFA, changing buffer B at 2%/min. For each mutein, the refolded protein eluted as a sharp peak approximately 2 minutes earlier than the reduced denatured but non-refolded proteins.

D. Purification of Refolded Muteins

The refold solution was concentrated approximately 10 fold with an Amicon S10Y3 membrane which has a 3kDa cutoff (Amicon division of WR Grace and Co., Beverly Massachusetts) and dialyzed into 20 mM sodium phosphate, pH 6.0. The dialyzed solution was centrifuged at 17,700 x g for 30 minutes and the supernatant was filtered through a 0.2 micron filter. The filtered protein was loaded at 20 ml/minute onto a Q-Sepharose anion exchange column (5x30cm, Pharmacia Biotech,Piscataway, NJ) previously equilibrated with 20 mM Sodium Phosphate pH 6.0. The bound protein was eluted with a linear gradient (5 column volumes) to 20 mM Sodium phosphate, 0.5M NaCl, pH 6.0 at a flow rate of 20 ml/min. 25ml fractions were collected. Each mutein eluted at approximately 0.2-0.25M NaCl. Fractions were analyzed on a C4 reverse phase

column (RP-4 1X250mm, Synchrom, Lafayette, IN) using the same conditions described above for monitoring refolding or by SDS-PAGE.

Fractions containing the refolded mutein (fractions eluting at 0.2 - 0.25M NaCl) were pooled and dialyzed into 20 mM Tris HCl, 1M NaCl, pH 7.5. The dialyzed material was loaded at 25 ml/minute onto a Butyl Sepharose (Supelco, Bellefonte, Pa) hydrophobic interaction column (5 x 8 cm Pharmacia Biotech,Piscataway, NJ) previously equilibrated with 20 mM Tris HCl, 1M NaCl, pH 7.5. The bound protein was eluted with a linear gradient (10-15 column volumes) to 20 mM Tris, 0 NaCl, 25% ethanol, pH 7.5 at a flow rate of 20 ml/min. Each mutein eluted as an asymmetrical peak at approximately 15-20% ethanol.

100μl aliquots of the protein-containing fractions (eluting at 15-20% ethanol) were analyzed on a C4 reverse phase column (RP-4 1X250mm, Synchrom, Lafayette, In) using the same conditions described above for monitoring refolding or by SDS-PAGE. Fractions containing purified muteins were pooled and concentrated to approximately 0.8 mg/ml and dialyzed into 20 mM Tris, 250 mM NaCl, pH 7.4. The purified refolded muteins were assayed for bioactivity as described in Example 9.

Example 8

PEGylation of IGFBP-1 Muteins

The C98 and C101 muteins were PEGylated using monomethoxy-PEG, with an average molecular weight of 20kDa, having a thiol-specific maleimide reactive group attached (CH_3O-$(CH_2CH_2O)_n$-$NHCOCH_2CH_2$-N), where n is the number of monomeric units. Preparation and use of other suitable PEG-maleimide reagents is discussed in PCT Application Publication No. WO92/16221, incorporated herein by reference.

During refolding, the substituted cysteine residue (CYS 98 or CYS 101 in C98 and C101, respectively) can form mixed disulfides with glutathione, to form Cys-S-S-GSH, or with cysteine, to form Cys-S-S-Cys. Accordingly, there may be no free thiol available for reaction with the PEG reagent. Therefore, the purified muteins were partially reduced prior to reaction with the PEG reagent.

The partial reduction was accomplished by reacting the purified mutein (0.45mg/ml) with DTT at a molar ratio of DTT to protein of 5.625 to 1 in 20 mM Tris, pH 7.4, 250 mM NaCl for 2 hours at room temperature. Reduction was stopped by acidification to pH 5.5. The DTT was removed by dialysis into 10 mM sodium acetate, pH 5.5.

The partially reduced muteins were each reacted with the PEG reagent, at a 4 to 1 molar ratio of PEG to protein (final protein concentration of 0.33 mg/ml) in 15mM sodium acetate, 26mM sodium phosphate, pH 7.0, 120mM NaCl for 4 hours at room temperature. SDS-PAGE analysis of the reaction mixture showed approximately 50% of the partially reduced muteins were converted to a mono-PEGylated species (C98-PEG, C101-PEG) having an approximate apparent molecular weight of about 67 kDa. The large apparent molecular weight was due in part to PEG interactions with the gel.

The reaction mixture was adjusted to 20 mM sodium phosphate pH 6.5. Q-A Q Sepharose anion exchange column (5 x 10 cm, Pharmacia Biotech, Piscataway, NJ) was equilibrated with 20 mM sodium phosphate pH 6.5 and the reaction mixture was loaded at at 20 ml/minute. The bound protein was eluted with a linear gradient (10 column volumes) to 20 mM sodium phosphate, 1M NaCl, pH 6.5 at a flow rate of 20 ml/min. Each of the C98 and C101 PEGylated muteins eluted at approximately 0.2 M NaCl. 25 ml fractions were collected and aliquots were analyzed by SDS-PAGE. Fractions containing PEGylated C98 or C101 which gave a single predominant band on non-reducing SDS PAGE corresponding to the PEGylated mutein were pooled and assayed for Bioactivity as described in Example 9.

Example 9

IGFBP-1 and its muteins inhibit IGF-1 stimulated growth of 3T3 cells

A crystal violet dye assay was used to measure cell proliferation. Assays were performed in 96 well gelatin-coated plates. Balb/c 3T3 fibroblasts were plated at 25,000 cells/well in 200 μl of serum-free DMEM (Dulbecco's modification of Eagle's media, Mediatech, Herndon, VA) and 0 - 850 ng/ml IGF-1. Cells were incubated for 72 hours at 37°C . At this time, the media was

replaced with 150 μl of 0.2% crystal violet, 10% formaldehyde, 10 mM potassium phosphate pH 7.0. After a 20 minute incubation at room temperature, the wells were washed 3 times with phosphate buffered saline ("PBS"), and the cell-bound dye released by incubation with 200 μl/well of 50% ethanol/0.1M sodium citrate, pH 4.2. Absorbance at 570 nm was read the next day. The results, set forth in Table 13, show that recombinant IGF-1 stimulates proliferation of 3T3 fibroblast cells in a dose dependent manner. Maximal proliferation occurred at a IGF-1 concentration of about 20-60 ng/ml. The ED_{50} was approximately 5-20 ng/ml.

The effect of IGFBP-1, the C98 and C101 muteins, and the PEGylated muteins on IGF-1-stimulated proliferation of 3T3 fibroblasts was determined by co-incubating the cells with a set amount of IGF-1 and increasing amounts of the binding proteins. Balb/c 3T3 fibroblasts were plated at 25,000 cells/well in 200 μl of serum-free DMEM containing 21ng/ml IGF-1, and varying amounts of IGFBP-1 and the various muteins (0 ng/ml - 11,520 ng/ml). The cells were incubated for an additional 72 hours and processed as described above.

The results show that the bioactivities of both the unPEGylated and PEGylated C98 and C101 muteins are comparable to the activity of wild type recombinant IGFBP-1 (Tables 14-18). The concentration of IGFBP-1 required to inhibit 50% of the activity of 21ng/ml IGF-I (IC_{50}) under the conditions described is approximately 200 - 300 ng/ml for wild type IGFBP-1, the IGFBP-1 muteins, and for PEGylated IGFBP-1.

TABLE 14

STIMULATION OF 3T3 FIBROBLAST GROWTH BY IGF-I

IGF-I (ng/ml)	Absorbance* (570nm)	S.E.**
850.00	1.587	0.076
212.50	1.627	0.053
53.13	1.662	0.039
13.28	1.495	0.029
3.32	1.112	0.037
0.83	0.920	0.019
0.21	0.915	0.020
0.05	0.897	0.014
0.01	0.862	0.011
0.00	0.781	0.016

* Absorbance values are the mean of 12 samples

** S.E. represents standard error of the mean.

TABLE 15.

Dose-dependent inhibition of 3T3 fibroblast growth by the C98 mutein

C98 mutein (ng/ml)	*Absorbance 570nm	**S.E.
11520.00	0.941	0.085
2880.00	1.001	0.033
720.00	1.161	0.028
180.00	1.580	0.013
45.00	1.775	0.023
11.25	1.787	0.006
2.81	1.797	0.016
0.70	1.747	0.011
0.18	1.818	0.004
0	1.800	0.016

* Absorbance values are the mean of triplicate samples

** SE represents standard error of the mean.

TABLE 16

Dose-dependent inhibition of 3T3 fibroblast growth by the C101 mutein

C101 mutein (ng/ml)	Absorbance 570nm	S.E.
11520.00	0.930	0.058
2880.00	1.032	0.033
720.00	1.108	0.052
180.00	1.413	0.052
45.00	1.656	0.057
11.25	1.620	0.039
2.81	1.641	0.064
0.70	1.593	0.015
0.18	1.584	0.050
0	1.516	0.052

Absorbance values are the mean of triplicate samples. SE represents standard error of the mean.

TABLE 17
Dose-dependent inhibition of 3T3 fibroblast growth by the C98 PEGylated mutein

C98-PEG mutein (ng/ml)	Absorbance 570nm	S.E.
11520.00	0.915	.015
2880.00	0.957	.019
720.00	1.049	0.044
180.00	1.302	0.034
45.00	1.473	0.028
11.25	1.595	0.031
2.81	1.620	0.028
0.70	1.581	0.019
0.18	1.585	0.015
0	1.699	0.024

TABLE 18
Dose-dependent inhibition of 3T3 fibroblast growth by the C101 PEGylated mutein

C101-PEG mutein (ng/ml)	Absorbance 570nm	S.E.
11520.00	0.956	0.041
2880.00	1.008	0.016
720.00	1.082	0.027
180.00	1.299	0.030
45.00	1.505	0.061
11.25	1.596	0.020
2.81	1.589	0.011
0.70	1.564	0.003
0.18	1.577	0.020
0	1.614	0.016

Absorbance values are the mean of triplicate samples. SE represents standard error of the mean.

TABLE 19

Dose-dependent inhibition of 3T3 fibroblast growth by wild type IGFBP-1

WT IGFBP-1 (ng/ml)	Absorbance 570nm	S.E.
11520.00	0.74	0.015
2880.00	1.799	0.036
720.00	1.015	0.010
180.00	1.213	0.026
45.00	1.351	0.045
11.25	1.432	0.048
2.81	1.475	0.074
0.70	1.538	0.020
0.18	1.530	0.039
0	1.583	0.022

Example 10

Pharmacokinetics of IGFBP-1 and PEGylated IGFBP-1

Four male Sprague Dawley rats were used to determine pharmacokinetic parameters. Two rats were injected with an I.V. bolus of 1mg/kg recombinant human IGFBP-1 and two rats were injected with an I.V. bolus of 1mg/kg of the PEGylated IGFBP-1 C101 mutein. The C101 mutein had been prepared and PEGylated as described in Examples 7 and 8. Tail vein blood samples were taken at 0.016, 0.083, 0.033, 0.075, 1.5, 2, 3, 5, 6, 8, 10, 12, 24, and 48 hours after the injection. The blood was collected in EDTA-coated tubes and centrifuged to collect the plasma fraction. The concentration of IGFBP-1 in the plasma samples was determined by ELISA using the Medix Biochemica (Kauniainen, Finland) IGFbp-1 test kit, Catalog No. 10831ETMB.

The plasma concentrations for the two rats in each test group were averaged and fit to a two or three exponential curve using Rstrip II (Micromath Software, Salt

Lake City, Utah). Data from the fitted curves appears in Table 20. ELISA-detectable plasma IGFBP-1 disappeared triexponentially and biexponentially after injection of the wild type IGFBP-1 and PEGylated C101 mutein, respectively.

Using the fitted curve, standard pharmacokinetic parameters were calculated as set forth in Pharmacokinetics, Gibaldi, M., and Perrier, D.; Swarbrick ed., 1975. These parameters appear in Table 21. The results show that PEGylation improves the pharmacokinetic performance of IGFBP-1 by increasing the circulation time because of decreased plasma clearance.

TABLE 20

Time/hr	Plasma IGFBP-1 (ng/ml)	Plasma PEG IGFBP-1 (ng/ml)
0.017	9057 ± 410	18120 ± 430
0.108	4937 ± 150	17060 ± 280
0.363	2032 ± 59	15340 ± 2000
0.751	1060 ± 32	14550 ± 1100
1.50	385.0 ± 35	12920 ± 12
2.03	261.0 ± 20	11590 ± 510
2.99	142.7 ± 0.85	10110 ± 150
4.97	39.84 ± 3.3	8503 ± 400
5.97	20.19 ± 1.8	7224 ± 590
7.97	ND*	4916 ± 250
9.97	ND	3196 ± 180
12.0	ND	2258 ± 130
26.0	ND	450.0 ± 30
50.0	ND	83.24 ± 1.2

*Not detectable

TABLE 21

parameter*	wild type IGFBP-1	PEGylated IGFBP-1
initial distribution volume, L/kg	0.095	0.063
steady-state distribution volume, L/kg	0.19	0.074
plasma clearance, mL/min/kg	6.2	0.15
fast (initial) half-life, min	2.9	
intermediate half-life, hr	0.24	3.9
slow (terminal) half-life, hr	1.1	13
plasma mean residence time, hr	0.52	8.2

*Mean values for two rats per group.

Example 11

IGFBP-1 inhibits restenosis in the rat

IGFBP-1 was tested for capacity to modify the proliferative response in carotid arteries of rats following balloon angioplasty. Nineteen Sprague Dawley rats weighing approximately 375g underwent surgery to implant jugular catheters in the right jugular vein. One week later the catheters were tested for patency and continuous saline infusion was initiated via a tethered infusion system. The tethered infusion system is set forth in Francis, P.C., et al., "Continuous Intravenous Infusion in Fisher 344 rats for Six Months: A Feasibility Study," Toxicology Methods, Vol. 2, pp.1-13 (1992), specifically incorporated herein by reference. Three days later, all animals had balloon angioplasty surgery via an arteriotomy incision in the left external carotid as set forth in Edelman, E.R. and Karnovsky, M.J., Circulation, Vol 89, No.2, pp. 770-776 (1994) specifically incorporated herein by reference. A 2F Fogarty balloon catheter (American Edwards Laboratories, Santa Ana, Calif.) was advanced to the aortic arch and pulled back with the balloon distended with sufficient air to generate resistance and denude the endothelium. This procedure was repeated 6 times to insure sufficient damage to the artery necessary to induce a proliferative response in the vessel wall. The external carotid was then ligated. The animals were divided into 2 groups and treatments were initiated immediately after angioplasty and

continued for 14 days. Group 1 (N=9) consisted of control animals which were infused IV with isotonic saline (0.25 ml/hr). Group 2 (N=10) animals were treated with IGFBP-1 by continuous IV infusion at a dose of 179μg/kg/hr. Blood samples were collected from the tail vein at 3, 9 and 14 days post angioplasty for determination of plasma levels of IGFBP-1. The concentration of IGFBP-1 in the plasma samples was determined by ELISA using the Medix Biochemica (Kauniainen, Finland) IGFbp-1 test kit, Catalog No. 10831ETMB. Infusions were discontinued at the time of blood collection (approximately 15 to 30 minutes).

At the termination of the study on day 14, catheter patency was confirmed by Brevital testing as set forth in Francis et al. The animals were anesthetized with a combination of Ace Promazine, Rompun and Ketamine and perfused with 10% neutral buffered formalin via cardiac puncture. Both carotid arteries were removed and placed in formalin for 2 additional days prior to processing for paraffin embedding. Three sections of carotid, encompassing almost the entire length of the lesion, were embedded from each animal to insure that all areas of the tissue were evaluated. Additional tissues (lung, heart, liver, kidney, spleen and adrenals) from all animals were collected for histologic evaluation to determine systemic effects (if any) of continuous infusion of IGFBP-1. The carotid artery sections were stained with hematoxylin and eosin, Masson's trichrome and toluidine blue. Other tissues were stained with hematoxylin and eosin only.

Mean plasma levels of IGFBP-1 at the termination of the study were 3.69 ± 0.64 μg/ml.

The effects of treating the animals with IGFBP-1 were determined by gross scoring, and by measurement of neointima (μm and pixels), neointima plus media (pixels), and media (pixels). The ratio of neointima (pixels) to media (pixels) was also calculated. Those scores and measurements appear in Table 22. Wilcoxon Rank-Sum (Mann-Whitney U) tests were performed for each of those six response measures and p-values determined using the table set forth in Natrella, M.G., Experimental Statistics, National Bureau of Standards Handbook 91, p. T-80 (1967). The p-values from the Wilcoxon Rank-Sum (Mann-Whitney U) tests are reported below. The data show that IGFBP-1 significantly reduces restenosis in the rat.

A gross assessment of treatment-related effects was done by assigning scores of 0, 1, 2, 3 or 4 to the lesioned carotids with 0=no observable thickening and 4=severe thickening. The mean (± standard error) scores from treated and control animals were 1.5 ± 0.27 and 2.67 ± 0.24, respectively. These values were found to be statistically significant ($p < 0.05$). In this gross assessment, the inhibition of thickening in the IGFBP-1 treated animals was 44%.

The neointima was also measured. The distance from the medial side of the neointima to the luminal side of the neointima was measured at 4 points opposite and perpendicular to each other and the average of 3 sections determined. The mean ± SE of neointima in μm for treated and control animals was 78.50 ± 10.68 and 139.33 ± 8.91, respectively ($p < 0.01$). This measurement also showed a 44% inhibition of thickening in the IGFBP-1 treated animals.

Image analysis using the Image 1 System (available from S & M Microscopy, Colorado Springs, CO) was used to determine the area of neointima plus media, neointima alone, and media alone for treated and control animals (3 sections/animal). The area (mean ± SE) of neointima plus media in pixels for treated and control animals was 25,160.30 ± 1817.42 and 35,271.11 ± 1,403.16, respectively ($p < 0.01$). Using this analysis, treatment with IGFBP-1 reduced the neointimal thickness by 29%. The mean area in pixels of neointima alone was 14,015.40 ± 1,834.24 for the treated animals and 23,119.11 ± 1,200.39 for the control animals or 39% inhibition of thickening in the IGFBP-1 treated animals ($p < 0.01$).

Finally, the ratio of neointima to media was calculated. This parameter also demonstrated the beneficial effect of treatment with IGFBP-1. The ratio in treated animals was 1.25 ± 0.17 and 1.91 ± 0.13 in the control animals. Using this ratio, inhibition of restenosis was 35% ($p < 0.05$). The ratio of neointima to media is generally the most accepted method of evaluating treatment-related effects in this rat model. Edelman and Karnovsky, Circulation, Vol. 89, No. 2 (1994).

TABLE 22

ANIMAL NO GROUP	GROSS AREA	INTIMA-μm	MED+INT PIXELS	INTIMA PIXELS	MEDIA PIXELS	INT:MEDIA
2C	3	168	38693	26473	12220	2.2
3C	2	87	28927	16199	12729	1.27
4C	4	147	36516	23357	13159	1.78
6C	2	161	33042	23822	9220	2.6
7C	3	141	43016	28659	15357	1.83
8C	2	127	33144	21367	11777	1.81
9C	3	168	35767	24672	11095	2.23
10C	2	114	31331	20312	11019	1.84
11C	3	141	37004	23211	13794	1.66
MEAN	**2.67**	**139.33**	**35271.11**	**23119.11**	**12263.33**	**1.91**
SE	**0.24**	**8.91**	**1403.16**	**1200.39**	**593.32**	**0.13**
21BP	2	121	29893	19613	10280	1.9
22BP	0	27	17316	4755	12561	0.37
23BP	2	74	23501	13717	9785	1.39
24BP	2	87	25148	13986	11161	1.25
25BP	2	87	29361	17851	11510	1.52
26BP	0	47	18330	8432	9898	0.83
27BP	2	34	17132	5676	11456	0.5
28BP	2	127	31113	20359	10754	1.88
29BP	2	94	30737	18021	12896	1.4
30BP	1	87	29072	17744	11328	1.5
MEAN	**1.50**	**78.50**	**25160.30**	**14015.40**	**11162.90**	**1.25**
SE	**0.27**	**10.68**	**1817.42**	**1834.24**	**327.23**	**0.17**

Although this invention has been described with respect to specific embodiments, it is not intended to be limited thereto and modifications made by those skilled in the art are considered to fall within the spirit and scope of the instant invention.

What is claimed is:

1. A method of treating a patient having an IGF associated condition comprising administering a therapeutically-effective amount of IGFBP-1 or a modified form thereof.

2. The method of claim 1 comprising administering IGFBP-1 to said patient.

3. The method of claim 2, wherein the patient is administered an amount of IGFBP-1 sufficient to reach a concentration of IGFBP-1 in the range from about 0.1 μg to about 300 μg per ml in the bloodstream of the patient.

4. The method of claim 1 comprising administering a modified form of IGFBP-1 to said patient.

5. The method of claim 4, wherein the modified form of IGFBP-1 is IGFBP-1 attached to a polymer.

6. The method of claim 5, wherein said polymer is polyethylene glycol.

7. The method of claim 4, wherein the modified form of IGFBP-1 comprises 2 IGFBP-1 molecules, wherein said first IGFBP-1 is attached to one end of the polymer and said second IGFBP-1 is attached to the opposite end of the polymer.

8. The method of claim 1, wherein the IGF associated condition is selected from the group consisting of breast cancer, colon cancer, lung cancer, ovarian cancer, osteosarcoma, glioma, liver cancer, rhabdomyosarcomas, restenosis, acromegaly, obesity, diabetic nephropathy and diabetic retinopathy.

9. The method of claim 8, wherein the IGF associated condition is breast cancer.

10. The method of claim 8, wherein the IGF associated condition is colon cancer.

11. The method of claim 8, wherein the IGF associated condition is osteosarcoma.

12. The method of claim 8, wherein the IGF associated condition is acromegaly.

13. The method of claim 8, wherein the IGF associated condition is restenosis.

14. A pharmaceutical composition comprising IGFBP-1 or a modified form of IGFBP-1 in an acceptable pharmaceutical carrier.

15. The pharmaceutical composition of claim 14, comprising IGFBP-1 in an acceptable pharmaceutical carrier.

16. The pharmaceutical composition of claim 14, comprising a modified form of IGFBP-1 in an acceptable pharmaceutical carrier.

17. The pharmaceutical composition of claim 16, wherein the modified form of IGFBP-1 is IGFBP-1 attached to an inert polymer chain.

18. The pharmaceutical composition of claim 17, wherein the inert polymer chain is polyethylene glycol.

19. The pharmaceutical composition of claim 16, wherein the modified form of IGFBP-1 is IGFBP-1 attached to opposite ends of an inert polymer chain.

20. A method of treating or preventing restenosis comprising administering, to a patient in need thereof, a therapeutically-effective amount of a protein capable of binding IGF.

21. The method of claim 20, wherein said protein is IGFBP-1.

22. The method of claim 21, wherein said protein is a modified form of IGFBP-1.

23. The method of claim 22, wherein said protein is IGFBP-1 attached to a polymer.

24. The method of claim 23, wherein the polymer is polyethylene glycol.

25. The method of claim 23, wherein the modified form of IGFBP-1 comprises 2 IGFBP-1 molecules, wherein said first IGFBP-1 is attached to one end of the polymer and said second IGFBP-1 is attached to the opposite end of the polymer.

26. The method of claim 1, wherein the IGFBP-1 is not phosphorylated.

27. The method of claim 21, wherein the IGFBP-1 is not phosphorylated.

28. The method of claim 26, wherein the IGFBP-1 is recombinantly produced in E. Coli.

29. The method of claim 27, wherein the IGFBP-1 is recombinantly produced in E. Coli.

30. An insulin-like growth factor binding protein (IGFBP) which is not phosphorylated and which is capable of inhibiting the activity of IGF.

31. The IGFBP of claim 30, wherein the IGFBP is recombinantly produced in E. Coli.

32. The IGFBP of claim 30, wherein said IGFBP is IGFBP-1 or a modified form thereof.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 94/03755

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 A61K37/02 C07K13/00 C12N15/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 A61K C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,89 09792 (SYNERGEN INC.) 19 October 1989 see page 1, line 12 - line 17; claims 1,3,6,7; figure 6 see page 4, line 8 - line 15	1-4,8, 14,15, 26,28, 30-32
X Y	WO,A,89 08667 (ERASMUS UNIVERSITEIT) 21 September 1989 see page 4, line 14 - line 24; claims 1,10 see page 9, line 13 - line 19	1-6,8,9, 14-18 7,10-13, 19-25, 27,29

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 August 1994	31 -08- 1994

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Ryckebosch, A

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,92 16221 (SYNERGEN, INC.) 1 October 1992 cited in the application see claims 1,2,4,19,20	7,19
Y	CHEMICAL ABSTRACTS, vol. 106, no. 7, 16 February 1987, Columbus, Ohio, US; abstract no. 47939k, J.V. TRICOLI ET AL. 'ENHANCED LEVELS OF INSULIN-LIKE GROWTH FACTOR MESSENGER RNA IN HUMAN COLON CARCINOMAS AND LIPOSARCOMAS.' page 441 ; cited in the application see abstract & CANCER RES. vol. 46, no. 12,1 , 1986 pages 6169 - 6173	10
Y	CHEMICAL ABSTRACTS, vol. 101, no. 17, 22 October 1984, Columbus, Ohio, US; abstract no. 149094v, J. BLATT ET AL. 'PRODUCTION OF AN INSULIN-LIKE GROWTH FACTOR BY OSTEOSARCOMA.' page 498 ; cited in the application see abstract & BIOCHEM. BIOPHYS. RES. COMMUN. vol. 123, no. 1 , 1984 pages 373 - 376	11
Y	EP,A,0 369 943 (SANDOZ AG) 23 May 1990 see page 4, column 5, line 2 - line 14; claims 1,12 see page 9, column 16, line 8 - line 41	12
Y	CHEMICAL ABSTRACTS, vol. 118, no. 17, 26 April 1993, Columbus, Ohio, US; abstract no. 166418x, M.J. KHORSANDI ET AL. 'REGULATION OF INSULIN-LIKE GROWTH FACTOR-I AND ITS RECEPTOR IN RAT AORTA AFTER BALLOON DENUDATION: EVIDENCE FOR LOCAL BIOACTIVITY.' page 622 ; cited in the application see abstract & J. CLIN. INVEST. vol. 90, no. 5 , 1992 pages 1926 - 1931	13, 20-25, 27,29

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International Application No
PCT/US 94/03755

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GROWTH REGULATION vol. 3, no. 1 , March 1993 , EDINBURGH, GB pages 37 - 40 J.I. JONES ET AL. 'HUMAN IGFBP-1 IS PHOSPHORYLATED ON 3 SERINE RESIDUES: EFFECTS OF SITE-DIRECTED MUTAGENESIS OF THE MAJOR PHOSPHOSERINE.' see page 37, right column, line 62 - line 63 -----	30-32

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/03755

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark : Although claims 1-13, 20-29 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 94/03755

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-8909792	19-10-89	AU-A-	1955388	03-11-89
		EP-A-	0418230	27-03-91
		JP-T-	3504597	09-10-91
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		EP-A-	0404812	02-01-91
		JP-T-	3504494	03-10-91
WO-A-9216221	01-10-92	AU-A-	1674292	21-10-92
		EP-A-	0575545	29-12-93
		NO-A-	933270	01-11-93
EP-A-0369943	23-05-90	AU-B-	623447	14-05-92
		AU-A-	4453689	07-06-90
		CA-A-	2002609	11-05-90
		JP-A-	3041096	21-02-91